Identification of HNPCC by molecular analysis of colorectal and endometrial tumors

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Abstract. Hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) is a dominantly inherited syndrome character-
ized by the development of colorectal cancer, endometrial cancer and other cancers and the presence of microsatellite instability
(MSI) in tumors. The Bethesda guidelines have been proposed for the identification of families suspected of HNPCC that require
further molecular analysis. We have evaluated the yield of MSI-analysis in a large series of Dutch families suspected of HNPCC.
We also analysed whether the loss of mismatch repair (MMR) protein detected by immunohistochemistry (IHC) of colorectal
cancer (CRC) and endometrial cancer correlated with the presence of MSI and/or a MMR gene mutation.

The results showed that the Bethesda criteria with a few modifications are appropriate to identify families eligible for genetic
testing. In addition, we found that MSI and IHC-analysis of CRC using antibodies against MLH1, MSH2, MSH6 and PMS2
proteins are equally effective for identifying carriers of the known MMR gene defects. However, as long as the role of other
putative MMR genes in hereditary CRC has not been elucidated, IHC-analysis cannot completely replace MSI. For this reason, we
prefer MSI-analysis as first step in families suspected of HNPCC. On the other hand, in families fulfilling the revised Amsterdam
criteria in which the probability of detecting a mutation is relatively high, we would recommend IHC as first diagnostic step
because the result might predict the specific underlying MMR gene mutation. MSI or IHC-analysis of endometrial cancer alone
was found to be less sensitive compared with these tests performed in colorectal cancer. Therefore, probably the best approach
in the analysis of this cancer is to perform both techniques.

The identification of HNPCC is important as it makes it possible to target effective preventative measures. Our studies showed
that MSI and IHC analysis of colorectal and endometrial cancer, are reliable cost-effective tools that can be used to identify
patients with HNPCC.

1. Introduction

Approximately 10–15% of patients with colorectal cancer (CRC) have a family history of CRC, and 5% of patients have early-onset (<45 yrs) CRC [1]. In the etiology of CRC in these cases, a combination of genetic and environmental factors is likely to play a role. In a small fraction of cases, genetic factors play a dominant role. The most common dominantly inherited colorectal cancer syndrome is hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome, heredi- tary colorectal-endometrial cancer syndrome), which is characterized by the development of colorectal, en- dometrial, and various other cancers at an early age [2]. This syndrome is due to a mutation in one of the fol- lowing DNA mismatch repair (MMR) genes: *MSH2, MLH1, MSH6,* and *PMS2*. HNPCC accounts for 1–5% of all cases of CRC [3].

The identification of individuals predisposed to CRC is important, as it makes it possible to target effec-

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tive preventative measures. A detailed family history is the simplest and most cost-effective way to identify hereditary CRC. As cancer is a common disease, the occurrence of CRC in several members of one family might be due to clustering by chance. Characteristics of hereditary forms of nonpolyposis CRC that might be helpful in the differential diagnosis from nonhereditary cases are an unusual early age of onset, the occurrence of multiple CRCs, and the combined occurrence of CRC with endometrial cancer or another HNPCC associated cancer (in an individual or family).

During the last decade great progress has been made in molecular genetics. Various genes responsible for the inherited forms of CRC have been identified, and genetic testing has been implemented in clinical practice on a large scale. Advantages of genetic testing are that the hereditary nature of the disease can be confirmed, and that, in families with an identified mutation, the carriers of a mutated gene can be differentiated from non-carriers. The latter can be reassured and refrain from further screening. Along with the development of techniques to identify mutated genes, new diagnostic tools such as microsatellite instability (MSI) analysis and immunohistochemistry (IHC) have also been introduced. These new tests have been proven to be useful to select patients suspected of HNPCC for MMR mutation analysis. This review addresses the question how clinical criteria and molecular pathology can be helpful in the diagnosis of HNPCC.

2. The importance of clinical criteria for HNPCC

Predisposed individuals from HNPCC families have a high lifetime risk of developing CRC (60–85%), endometrial cancer (30–50%), as well as certain other cancers (below 15%) [4–6]. CRC is often diagnosed at an early age (mean 45 years), can be multiple (with synchronous or metachronous CRC present in 30% of patients), and, in about two-thirds of the cases is located in the proximal part of the colon. Microscopic features frequently observed in colorectal cancer associated with HNPCC are the presence of peritumoral and tumor infiltrating lymfocytes [7]. Extracolonic cancers observed in HNPCC include cancer of the endometrium, renal pelvis/ureter, stomach, small bowel, ovary, brain, hepatobiliary tract and sebaceous tumors.

The diagnosis of HNPCC is hampered by the absence of specific diagnostic clinical features. Therefore, in 1990, the international collaborative group on HNPCC (ICG-HNPCC) proposed a set of clinical diagnostic criteria (the Amsterdam criteria) in order to provide a basis for collaborative studies and to provide uniformity in the terminology of HNPCC [8]. Since then, many studies have shown that HNPCC is also associated with several other extracolonic cancers. That was the reason to propose a new set of criteria (the Amsterdam criteria II) (Table 1), which include various extracolonic cancers [9]. It should be noted that the proposed criteria were not intended for use as exclusion criteria, in other words, families initially suspected of HNPCC but not meeting the criteria should not be falsely reassured and excluded from genetic counselling, genetic testing or surveillance. On the other hand although in most families that meet the Amsterdam criteria there is evidence for a MMR defect, in some families such features (MSI and loss of MMR protein expression) are lacking. In these families, which are generally characterized by a relatively high mean age of onset of CRC and the absence of endometrial cancer, we are probably not dealing with HNPCC and it is questionable whether the HNPCC guidelines for surveillance (e.g. surveillance of endometrium) are applicable in such families.

In 1996, at an NCI workshop clinical criteria were proposed for families suspected for HNPCC that require further molecular analysis (Table 1) [10].

3. Results of mutation analysis of families suspected of HNPCC

The identification of the gene defects that are responsible for HNPCC almost ten years ago allowed making a presymptomatic diagnosis. However, due to the heterogeneity of the mutation spectrum in MMR genes, screening for mutations is both time-consuming and expensive. At our centre, mutation analysis has been performed in 287 research families not selected by genetic analysis of the tumour. Mutations were found in 30% of the families: 13% harboured a *MSH2* mutation, 14% a *MLH1* mutation and 3% a *MSH6* mutation. The mutation detection rates in families meeting the Amsterdam criteria I and those meeting the Amsterdam criteria II were the same (50%). In general, in the Netherlands, mutation analysis in families with hereditary cancer is performed if the predicted probability of detecting a mutation is 1 in 10 or higher. This means that the maximum costs of finding one pathogenic mutation is ten times the costs of testing one person (genetic counselling i.e. 1700 Euro and mutation analysis of three genes i.e. 3 × 620 Euro) that equals 35.600 Euro. Fortunately, in contrast to other hereditary can-

Table 1
Clinical criteria for suspected and probable HNPCC

Suspected HNPCC[1]	Probable HNPCC[2]
1. Patients with multiple synchronous or metachronous HNPCC-related cancers[3]	– At least three relatives with CRC, cancer of endometrium, small bowel, ureter, or renal pelvis
2. Patients with CRC and a first-degree relative with an HNPCC-associated cancer and/or adenoma, one of them aged <45 years (adenoma <40 years)	– One of the three is a first-degree relative of the other two
3. Patients with CRC or endometrial cancer at age <45 years	– At least two consecutive generations affected
	– Cancer diagnosed at age <50 years in at least one relative
4. Patients with adenomas < 40 years	– Histological confirmation of cancer diagnosis

[1]Original Bethesda criteria omitting the Amsterdam criteria.
[2]Amsterdam II criteria.
[3]CRC, endometrial, gastric, hepatobiliary, small-bowel, transitional cell cancer of renal pelvis or ureter.

Table 2
Costs of mutation analysis of MMR genes, MSI and IHC analysis and genetic counselling in the Netherlands

Type of test	Costs in Euro
Mutation analysis three genes	1860,-
MSI-analysis	620,-
IHC-analysis (four proteins)	400,-
Genetic counselling	1700,-

cers, in familial CRC cheaper tests, i.e. MSI and IHC-analysis, are available that can be used to identify families which have a high predicted probability of carrying a mutation. The costs of MSI and IHC-analysis, mutation analysis and genetic counselling in the Netherlands are shown in Table 2.

4. Selection of families for mutation analysis on the basis of micrsatellite instability (MSI) and immunohistochemical analysis (IHC)

Microsatellite instability (MSI) first reported in 1993 is caused by a failure of the DNA mismatch repair (MMR) system [11–13]. Microsatellites are repetitive DNA sequences found throughout the genome. Loss of MMR function may result in mutations in repetitive coding and noncoding regions of genes including those genes involved in tumor initiation and progression. According to international guidelines for the evaluation of MSI, a panel of five microsatellite markers should be used [10]. If two of the five markers show instability, the tumor is referred to as MSI-high (MSI-H). If one of the markers shows instability the tumor is considered MSI-low (MSI-L). A tumor without any instable marker is designated MS-stable (MSS). If in addition to the recommended set other markers are used, the tumor is referred to as MSI-H when 30% or more of the markers show instability. If less than 30% of the markers are unstable the tumor is considered as MSI-L. Because over 90% of colorectal cancers from patients

with HNPCC express a high level of MSI, MSI may aid in the diagnosis of this syndrome [3]. However, MSI is not specific to HNPCC, as it also occurs in 15% of apparently sporadic colorectal and other tumors. It has been recommended that MSI analysis should be performed in all tumors from patients that belong to families that meet the Bethesda criteria (Table 1) [10].

Another recently introduced rapid and cheap technique to identify mismatch repair deficiency is that of immunohistochemistry (IHC) of the MMR proteins in tumors. Wilson and Leach reported the use of antibodies against the MSH2 protein [14,15]. Thibodeau analysed the protein expression of *MLH1* and *MSH2* in sporadic CRC, familial CRC and CRC associated with HNPCC [16]. Later also the use of antibodies against the MSH6 protein was described.

We have recently evaluated the yield of MSI-analysis in a large series of Dutch families with proven or suspected HNPCC. We also analysed whether loss of MMR protein expression detected by immunohistochemistry correlated with the presence of MSI and/or a MMR germline defect. Here follows a summary of our findings.

5. Results of MSI-analysis in families that meet the Bethesda criteria

Various studies have shown that the Bethesda criteria are appropriate to identify HNPCC families [17,18]. We have recently performed MSI-analysis in 725 individuals from 631 families suspected of HNPCC. We used in addition to the five microsatellite markers recommended by the NCI workshop three mononucleotide repeat markers (BAT 40, MSH3 and MSH6) because in our experience these markers, especially BAT 40, increased the sensitivity of the test [19]. Tumors were classified as MSI-H when >30% of the markers showed instability, as MSI-L if <30% of the markers showed

instability. We distinguished between MSI-L with instability of a dinucleotide marker only (MSI-Ld) and instability of a mononucleotide marker only (MSI-Lm) because we have found that MSI-Lm is more informative for true MMR deficiency than MSI-Ld. A MSI-H or MSI-Lm phenotype was observed in 39% of the patients from families that meet the Bethesda criteria [20]. The proportion of MSI-H /MSI-Lm tumors was highest in families that met the Amsterdam criteria (59%) and in patients with multiple HNPCC associated cancers (44%). Our database of 631 families included also 195 families that were suspected of HNPCC but that did not fulfill the Bethesda criteria, i.e., families with solitary patients with CRC diagnosed between age 45 and 50 years; families with two first-degree relatives with CRC and HNPCC-cancer, respectively, one diagnosed between age 45 and 50 years; and families with three first-degree relatives with CRC all diagnosed >50 years. Also in these subsets of families a substantial proportion (>19%) showed a MSI-H or MSI-Lm phenotype. On the basis of our results we recommend to extend the Bethesda criteria and to perform MSI-analysis also in families that meet the latter criteria.

6. Comparison of MSI and IHC-analysis

In order to compare the effectiveness of MSI-analysis and IHC-analysis to identify families with HNPCC, we evaluated the results of these tests in a large series of proven carriers of a pathogenic mutation and in a large series of families suspected of HNPCC [19–21]. In 12 patients with a *MSH2* mutation, all colorectal tumors had an MSI-H/L phenotype as well as loss of staining of at least one MMR protein [19]. Analysis of colorectal tumours from 21 *MSH6* mutation carriers showed in all cases a MSI-H or MSI-L phenotype. Immunohistochemical analysis of these tumors showed loss of staining of at least one MMR-protein in all but one case [21]. MSI-analysis of tumors from 21 *MLH1* mutation carriers showed a MSH-H or MSI-L phenotype in 93% of the cases. However, with IHC-analysis using antibodies against MLH1, MSH2 and MSH6 we missed 3 out of 21 cases [19]. Because the PMS2 protein forms a heterodimer with the MLH1 protein, we hypothesized that absence of the MLH1 protein due to a germline mutation also leads to loss of PMS2 protein caused by abrogation of the total protein complex. Indeed using antibodies against PMS2 in an extended series of tumors from 35 carriers of a *MLH1* mutation, loss of both proteins was frequently observed and by adding PMS2

antibodies significantly more (23%) *MLH1* mutation carriers were identified [20]. Data on MSI-analysis as well as IHC staining (4 proteins) were available for 284 tumors of patients from families suspected of HNPCC. Among these 284 tumors, 91 showed MSI-H as well as abnormal staining and 136 tumors showed MSS and normal protein expression, leading to concordant results in 93% of the MSI-H and MSS tumors. Among tumors with an MSI-L phenotype, those with instability of a mononucleotide marker (MSI-Lm) showed more often absence of an MMR-protein (35%) than a MSI-L tumor with one instable dinucleotide marker (MSI-Ld) (13%). In conclusion, MSI and IHC-analysis using antibodies against MLH1, MSH2, PMS2 and MSH6 appear to be equally effective for the identification of mutation carriers.

7. MSI and IHC- analysis in adenomas from carriers of MMR gene mutation

According to the Bethesda guidelines, the finding of adenomas in an individual before age 40 years is an indication for MSI-analysis. A large Finnish study of apparently sporadic adenomas have shown that 1.6% (6/378 adenomas) have a MSI-H phenotype [22]. Five of the six patients with a MSI-H adenoma were found to carry a MMR mutation. Another recent study showed that about 75% of adenomas detected in HNPCC families showed MSI as well as loss of MMR protein [23]. We recently compared the features of adenomas in a large series of HNPCC mutation carriers and their relatives who were found not to carry a mutated gene (controls) [24]. The adenomas in carriers were larger, and a higher proportion had villous components and/or high grade dysplasia ($P < 0.05$ in all cases). Moreover, in most of the adenomas (74%) of the mutation (*MSH2* or *MLH1*) carriers, IHC-analysis showed absence of at least one MMR protein. Based on these findings we recommend IHC-analysis (or MSI-analysis) of adenomas detected in young patients (<50 yrs) if the adenomas are large (>7 mm), show high-grade dysplasia and/or have a villous component.

8. MSI and IHC-analysis in endometrial cancer

Endometrial cancer is the most common extracolonic cancer in women with HNPCC. In some families suspected of HNPCC only tumor tissue is available of endometrial cancer. An important question is therefore

Table 3
Consequences of test-results on clinical management of families that comply with the Bethesda or Amsterdam criteria

Clinical criteria	MSI -analysis	IHC-analysis	Mutation analysis	Clinical management
Amsterdam criteria	Two MSS tumors	Normal protein expresssion in two tumors	Not indicated	Colonoscopy 1x/3–5 years from 5–10 years before first diagnosis CRC in family
Bethesda criteria[1]	MSI-H/L	Loss of MSH2/MSH6 or MSH6	No mutation identified	According to HNPCC guidelines[2]
Bethesda criteria[1]	MSI-H/L	Loss of MLH1	No mutation identified	According to HNPCC guidelines if additional features[3] suggest a HNPCC cancer

[1]excluding the Amsterdam criteria.
[2]surveillance by colonoscopy every two years from age 20–25 years, annual surveillance of endometrium from age 30 years; consider subtotal colectomy and ileorectal anastomosis in patients that present with CRC.
[3]young age, pathology features (tumor heterogeneity, peritumoral lymphocytes, tumor-infiltrating lymphocytes), absence of methylation of MLH1 promotor.

whether MSI and IHC analysis are equally effective to identify HNPCC in endometrial cancer as in CRC. Two recent studies have shown that MSI and loss of MMR protein can be observed in about 35% of patients with endometrial cancer diagnosed before age 50 years [25,26]. In one study, a MMR gene mutation was only identified in endometrial cancer patients who had a first-degree relative with an HNPCC-associated cancer [26]. In the other study, a substantial proportion of patients without a personal or family history of an HNPCC associated cancer had a MSI-H endometrial cancer [25]. In the latter study, no information was provided on the mutation status of the patients.

We have analysed the sensitivity of MSI and IHC in endometrial tumors from family members with a known mutation in *MLH1, MSH2* and *MSH6* [27]. We have shown that all endometrial tumors from *MLH1/MSH2* mutation carriers demonstrated a MSI-H phenotype, while in endometrial tumors from *MSH6* carriers only one third demonstrated a MSI-H phenotype. On the other hand, in tumors from *MSH2/MSH6* mutation carriers, IHC predicted the presence of a *MSH2* or *MSH6* mutation in all cases. However, in tumors from *MLH1* mutation carriers, IHC (using antibodies against MLH1, MSH2 and MSH6) could predict the mutation in *MLH1* in only 30% of the cases. Therefore, the best approach in families suspected of HNPCC with only endometrial cancer tissue available for analysis is to perform both MSI-analysis as well as IHC. Future studies should evaluate whether the use of PMS2 antibodies will lead to the identification of more *MLH1* mutation carriers. Also more studies are needed to evaluate the value of MSI/IHC- analysis in young (<50 years) endometrial cancer patients without a personal and/or family history of an HNPCC-cancer.

9. Conclusions

The results of our studies showed that both MSI-analysis as well as IHC-analysis of colorectal tumors is a sensitive screening method to identify patients eligible for mutation analysis. Although our studies indicated that the sensitivity of IHC is approximately the same as that for MSI, IHC cannot completely replace MSI analysis as long as the role of other putative MMR genes in hereditary CRC has not been elucidated. For this reason, we prefer MSI-analysis as first step in families suspected of HNPCC but not fulfilling the Amsterdam criteria. In these cases the probability of detecting a mutation is relatively low (<25%). In the total group of Amsterdam-negative families, MSI analysis will provide global information on loss of MMR function, including alterations in MMR genes other than the known genes. In MSI-H and MSI-L cases (if the unstable marker is a mononucleotide), IHC should be performed as second step. In the cases of MSS, IHC for MSH6 might be considered as one study had shown that tumors from *MSH6* carriers might be stable (MSS) [28]. On the other hand in families fulfilling the revised Amsterdam criteria in which the probability of detecting a mutation is relatively high (>50%), we would recommend IHC as first diagnostic step because the result might indicate which MMR gene is mutated. If a negative staining pattern is found, mutation analysis of the respective gene is the next step. In case of doubtful interpretation or positive staining of all MMR proteins, MSI analysis should be performed. In case of the absence of MSI, the analysis of a second tumor from the same family is recommended to exclude the possibility of phenocopies.

Various studies have shown that MSI and IHC analysis of endometrial cancers are also effective for the

identification of HNPCC. Our studies indicated that the best approach is to use IHC-analysis as well as MSI-analysis in endometrial cancer from members of families suspected of HNPCC. MSI and IHC analysis should also be performed in young patients with adenomas, which are large (>7 mm) and/or show high degree of dysplasia and/or a villous component.

An important question is what are the clinical consequences for relatives of patients with tests results (MSI/IHC) suggesting the presence of an MMR gene mutation but without identifying an MMR gene defect. There is no doubt that if a patient from a family that meets the Bethesda criteria has a tumor showing a MSI-H phenotype and loss of MSH2/MSH6 protein, he/she should be considered as HNPCC also if a germline *MSH2* or *MSH6* mutation cannot be identified. However, a more difficult question is whether a patient from a Bethesda positive family with a MSI-H CRC diagnosed at an intermediate age (50–65 years), loss of staining of MLH1 but without an *MLH1* mutation, should also be considered as an HNPCC patient? The question is whether the cancer has occurred sporadically or in the setting of an underlying germ-line mutation (HNPCC). Australian investigators have addressed this issue by examining the histopathological features, molecular changes, alteration in the immuno-histochemical profile, methylation, and patient age and sex in a large series of patients with either a sporadic MSI-H CRC or a HNPCC cancer [29]. Although they were not able to separate the two types of cancer into non-overlapping groups, and found that no single assay can be used to unequivocally partition of the groups, the combination of age at diagnosis and three pathology features (tumor heterogeneity, peritumoral lymphocytes, and tumor-infiltrating lymphocytes) allowed most of MSI-H cancers (>90%) to be classified as sporadic or HNPCC. If the cancer is classified as HNPCC, then the first-degree relatives should be screened according to the HNPCC guidelines (colonoscopy every two years starting from age 20–25 years) (Table 3).

At present, MSI and IHC-analysis are advised only in families that comply with specific criteria. Because, there is increasing evidence that MSI is an important prognostic factor and may predict the response to chemotherapy, in the near future these tests might be performed on a much larger scale if not in all CRC cases [30,31].

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